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PILOT SCALE PURIFICATION OF α-GALACTOSIDASE A FROM COHN FRACTION IV-1 OF HUMAN PLASMA

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Summary

Human plasma α-galactosidase A (α-D-galactoside galactohydrolase, EC 3.2.1.22) was purified 7000-fold over plasma levels from Cohn Fraction IV-1. The yield per kg starting material averaged 11 000 units (nmol galactose liberated per h) and the specific activity was about 600 units per mg protein with 4-methylumbelliferyl-α-D-galactoside. The ratio of 4-methylumbelliferyl-α-galactosidase to ceramide trihexosidase activities was 6.2. Both activities were heat labile and exhibited the same relative mobilities on polyacrylamide gel electrophoresis. Enzymatic activity was stable for at least 4 months at 4 and −20°C. The endotoxin concentration of this preparation averaged 0.26 mg per mg protein.

Introduction

Fabry's disease is an inborn error of glycosphingolipid metabolism resulting from the deficiency of a thermolabile lysosomal enzyme, α-galactosidase A (α-D-galactoside galactohydrolase, EC 3.2.1.22) [1—4]. Three kinds of glycosphingolipids, particularly globotriglycosylceramide (ceramide trihexoside), accumulate in the cardiovascular system and kidney and, to a lesser extent, in other tissues as a result of this disorder [4—9]. These glycosphingolipids all

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Abbreviations: 4-Mu-Gal, 4-methylumbelliferyl-α-D-galactopyranoside; $GbOse_3Cer$, globotriglycosylceramide; TEMED, *N,N,N',N'*-tetramethylethylenediamine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

possess terminal α-galactosidic linkages (see review, ref. 4).

Interest in the purification and characterization of α-galactosidase A has been stimulated by the possibility that enzyme replacement therapy might be an effective approach for control of Fabry's disease. The enzyme is widely distributed in normal tissues [4,10] and has been prepared from several sources [4,10,11].

This paper presents a method for the partial purification of human plasma α-galactosidase A on a pilot scale from Cohn Fraction IV-1. Some properties of the final product are described and evidence is given that it may be used safely for studies of enzyme replacement therapy.

Materials and Methods

Source of α-galactosidase. Plasma α-galactosidase A activity was recovered primarily in fraction IV-1 by the Cohn (Method 6) procedure [12]. This material was obtained from the Michigan Department of Public Health Blood Fractionation Laboratory in 6—9 kg batches of frozen, yellowish-green paste, which had been produced from outdated plasma. It was stored at −20°C until used.

Materials and chemicals. Whatman DE-52 and CM-52 cellulose were purchased from Scientific Products, McGraw Park, Ill., U.S.A. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A.

Globotriglycosylceramide ($GbOse_3Cer$) was purified from pig intestine, labeled with ^3H and supplied by K. Dean [10]. The substrate 4-methylumbelliferyl-α-D-galactopyranoside (4-MU-Gal) was produced by Koch-Light, Colnbrook, Bucks, U.K. and obtained through Research Products International, Elk Grove Village, Ill., U.S.A.

Acetonitrile, non-spectral grade, was purchased from Burdick and Jackson Laboratories, Inc., Muskegon, Mich., U.S.A.; ethylene diamine was from Aldrich Chemical Co., Inc., Milwaukee, Wisc., U.S.A.; Fluram (Roche Diagnostics) was obtained from Pierce Chemical Co., Rockford, Ill., U.S.A.; human serum albumin (crystallized and lyophilized) was from Sigma Chemical Co., St. Louis, Mo., U.S.A.; injectable saline and sterile water were purchased from Cutter Laboratories Inc., Berkeley, Calif., U.S.A.; and sodium taurocholate was purchased from Calbiochem, San Diego, Calif., U.S.A. All other chemicals were of analytical reagent grade.

Dialysis (Model c/HFD-15) and ultrafiltration (Model b/HFU-1) units were obtained from Dow Chemical Co., Midland, Mich., U.S.A. Sterile filtrations were made with a filter (Acrodisc 4192) from Gelman Instrument Co., Ann. Arbor, Mich., U.S.A. The final product was filtered and stored in a sterile, disposable 150 ml filter unit (0.45 μm; No. 7102; Falcoln, Oxnard, Calif., U.S.A.). Sephadex chromatography was carried out with a column (K 50/100) from Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A.

Polyacrylamide gels were formulated with acrylamide, bisacrylamide, tetramethylethylenediamine (TEMED) and ammonium persulfate from Ames Co., Elkhart, Ind., U.S.A. Behring immunodiagnostic reagents were obtained from American Hoechst Corp., Somerville, N.J., U.S.A.

Enzyme assays. Estimations of α-galactosidase activity were made by the

procedure of Desnick et al. [13], with the following modifications. An artificial substrate was used and all volumes were reduced by one-half. An Aminco Fluorocolorimeter (Model J4-7439, American Instrument Co., Silver Spring, Md., U.S.A.) was used with a Corning 7-60 primary filter and Wratten 2A and 48 secondary filters for fluorescence measurements. The reaction mixture contained 39 mM Na_2HPO_4/23 mM citric acid (pH 4.6), 4.3 mM 4-MU-Gal and 25 μl enzyme solution in 175 μl final volume. The reaction was terminated with 2.3 ml 0.1 M ethylene diamine. One unit of enzyme activity is that amount which hydrolyzes 1 nmol of artificial substrate per h at 37°C.

Estimations of α-galactosidase activity with a glycosphingolipid substrate were made with ^{3}H-labeled $GbOse_3Cer$ by the method of Dean and Sweeley [10], with the following modifications: the citrate buffer was replaced with 0.1 M sodium acetate (pH 3.8) since higher (40%) measured activities were obtained. The specific activity of the substrate was increased to 22 800 cpm/nmol and 50 000 cpm was used in each assay. The observed radioactivity in the upper phase was corrected to that expected for 0.5 mM substrate with the Michaelis-Menten equation (average literature K_m = 0.45 mM). This correction was found to be reasonably accurate provided the reaction consumed less than 10% of the substrate. Reaction mixture contained 50 mM sodium acetate (pH 3.8), 9.3 mM sodium taurocholate, 22 μM $GbOse_3Cer$ (50 000 cpm) and 20—40 μg enzyme in 100 μl final volume. One unit of enzyme is that amount which hydrolyzed 1 nmol of natural substrate per h at 37°C.

Protein determinations. Protein concentrations were estimated by a fluorescamine method [14]. A stock solution of fluorescamine was prepared by adding 200 ml of dry acetonitrile to 60 mg Fluram in a dry reagent bottle. The stock solution was stable at room temperature for at least 2 months, in the absence of H_2O.

The protein sample (5—50 μl) was added to 1.0 ml 0.2 M sodium borate (pH 9.0). While mixing vigorously on a Vortex-type mixer, 0.35 ml fluorescamine reagent was rapidly added and the relative fluorescence was determined. A standard curve, based on the fluorescence obtained with human serum albumin, was determined with each set of samples, using a stock solution containing 25 mg albumin in 100 ml 25 mM sodium phosphate (pH 6.0).

Pyrogen determinations. Levels of pyrogen (endotoxin) in the water supply, buffers, and intermediate fractions in the isolation of plasma α-galactosidase were determined with a Pyrostat Limulus reagent (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). The instructions from Worthington were followed, except that 0.5 ml, reagent and 50 μl sample were mixed in smaller tubes and absorbances determined in microcuvettes.

Purification of plasma α-galactosidase A. All steps were performed at 4°C, using glass-distilled water with a low pyrogen content (<0.3 ng/ml). Measurements of pH were made at 4°C with a meter that was calibrated at 25°C. The α-galactosidase activity with 4-MU-Gal substrate was quite stable at room temperature but the purification was carried out at 4°C to minimize bacterial contamination. The enzyme was unstable above pH 7.5 or below pH 4.5.

Frozen Cohn fraction IV-1 (6.4 kg) was added to 30 l of 0.025 M sodium phosphate buffer (pH 6.0) and was mixed until a smooth suspension was obtained. After 30 min, the pH was adjusted to 5.4 with 1 M NaOH. Stirring

was continued for an additional 30 min, after which the suspension was centrifuged (Sorvall RC-2B, GS-3 rotor) at 11 000 $\times g$ for 10 min. The precipitate was discarded and the pooled supernatant fractions were adjusted to pH 6.0 with 1 M NaOH.

The crude extract was mixed in two batches with a total of 15 l (settled volume) of regenerated DEAE-cellulose for 30 min. The mixture was then filtered in a Buchner funnel and suspended in 20 l of 0.025 M sodium phosphate buffer (pH 6.0)/0.030 M NaCl. The mixture was stirred for 15 min and then filtered as before. The resulting solid was suspended in 10 l phosphate buffer (pH 6.0)/0.035 M NaCl and the mixture was poured into a Pyrex glass column. After settling for about 5 min, the outlet was opened and the excess liquid drained from above the column packing. The column was washed with an additional 12 l of the second phosphate/chloride buffer. The entire column was a uniform blue-green color. The enzyme was eluted with a 30 l linear gradient of 0.038—0.120 M NaCl in 0.025 M sodium phosphate (pH 6.0) (flow rate, 4 l/h). Fractions were collected and α-galactosidase activity determined with 4-MU-Gal. The α-galactosidase activity eluted as a broad peak at about 0.05 M NaCl. Fractions with a specific activity greater than 4 units/mg protein were combined.

The pooled DEAE-cellulose fraction was mixed with 1 vol. water and applied to a Pyrex glass column containing 4 l (settled volume) of DEAE-cellulose. The blue ceruloplasmin was adsorbed in a tight band at the top of the column and a broader, yellowish band extended about 1/3 down the column. The enzyme was eluted with a 15 l linear gradient of 0.038—0.120 M NaCl in 0.025 M sodium phosphate buffer (pH 6.0, flow rate, 3.5 l/h). Fractions with greater than 5 units/mg protein were combined. Collection was always made one or two fractions before the blue ceruloplasmin began to elute. The total protein in the pooled fraction from the second DEAE-cellulose column was 35—45 g.

A plexiglass column was filled with CM-cellulose and washed with 4 l cold 0.025 M citrate/phosphate buffer (9.6 mM citric acid, 14.1 mM Na_2HPO_4) (pH 4.35 adjusted at 25°C)/0.05 M NaCl (flow rate, 120 ml/min). The pooled fraction from the second DEAE-cellulose column was mixed with 0.5 vol. cold water and the pH was adjusted to 4.30 at 5°C with 0.67 M H_3PO_4 (citric acid was not used since it caused the enzyme and other proteins to precipitate). After sample application, which gave a yellow band 4—5 cm deep, the enzyme activity was eluted with a 22 l linear gradient of 0.05—0.120 M NaCl in citrate/phosphate buffer (pH 4.35). The fractions were assayed immediately and those with greater than 100 units/mg protein were pooled and the pH adjusted to 6.0 with 1 M NaOH.

Hollow-fiber units were used to concentrate the enzyme, from 6—8 l to 25—30 ml, in two stages. Before and after use they were washed with at least 1 ml of water per cm^2 of fiber surface area and were stored in 1.5% formalin (0.56% formaldehyde) at 4°C. The overall recovery of activity from the two concentration steps was 90—99%. The sample was clarified by centrifugation at 15 000 $\times g$ for 20 min and sterile filtered through a 0.22 μm Sweeney filter.

A 5 cm column was packed with autoclaved Sephadex G-200 (40—120 μm diameter), equilibrated in sterile 0.025 M sodium phosphate (pH 6.0)/154 mM NaCl. The column was eluted in an upward flow direction at 20—25 ml/h. Frac-

tions with a specific activity greater than 400 units/mg protein were combined. The product was filtered and stored in a sterile container.

Fractions from 2—4 first Sephadex G-200 runs were routinely pooled, concentrated with a hollow-fiber unit, and re-run on Sephadex G-200. This step typically gave an additional 2-fold purification, with 75—85% recovery of α-galactosidase activity. Material from 19 batch runs was purified through this step. The entire product was then concentrated to 142 ml, dialyzed in the hollow-fiber beaker unit against sterile buffer (120 mM NaCl, 25 mM sodium phosphate, pH 6.0), sterile filtered and packaged under sterile conditions for use in subsequent studies of enzyme replacement therapy of patients with Fabry's disease.

Immunochemistry. Immunoelectrophoresis against rabbit whole anti-human sera was carried out according to a standard technique [15] with a detection limit of 0.2 mg/ml antigen. Radial immunodiffusion against anti-IgA, IgG, albumin, transferrin and ceruloplasmin was carried out according to the procedure of Mancini et al. [16]. Immunodiagnostic reagents gave a detection limit of 0.05 mg/ml antigen.

Polyacrylamide gel electrophoresis. In order to retain enzyme activity at pH 6.5, a new polyacrylamide gel system was formulated. The gel monomer stock contained 31.0 g acrylamide, 1.0 g bisacrylamide and water to 100 ml. The pH 6.0 gel buffer contained 11 ml 5 M HCl, 17.1 g sodium cacodylate, 0.43 ml TEMED and water to 100 ml. The electrolyte buffer was 1.3 g sodium cacodylate, 9.52 g HEPES and water to 11. These solutions were filtered (0.45 μm pore size) and stored at 4°C until use. The freshly prepared catalyst solution contained 14 mg ammonium persulfate per 10 ml water.

The gels were prepared from a solution of 1.5 parts gel monomer, 0.5 part gel buffer, 2 parts water and 4 parts catalyst.

Results

Pilot scale purification of plasma α-galactosidase activity

Human plasma α-galactosidase was purified over a 5 month period from approx. 130 kg Cohn fraction IV-1 from outdated plasma. There was no improvement in yield or purity with Cohn fraction from freshly frozen plasma. Better results were obtained when IV-1 paste was stored for less than 6 weeks. The procedure was begun on a new batch of Cohn fraction IV-1 (6.4 kg) every week and required about 12 working days to complete. The results of analyses at each step in the procedure for one batch are summarized in Table I.

Reproducible extraction of α-galactosidase activity from frozen IV-1 paste was achieved by adjusting the pH to 5.4. When the extraction mixture was adjusted immediately to pH 6.0, it was impossible to obtain a firm pellet upon centrifugation. Furthermore, the turbid supernatant fraction had a lower specific activity because more protein was extracted, although the total recovered α-galactosidase activity was higher at this pH.

Batch adsorption on DEAE-cellulose was used to circumvent problems of slow flow-rates, channeling, and poor reproducibility due to particulate matter in the crude extract. The NaCl concentration used to prewash the DEAE-cellulose before eluting the α-galactosidase was chosen to achieve maximum elution

TABLE I

PREPARATION OF α-GALACTOSIDASE FROM 6.4 kg OF COHN FRACTION IV-1

The data are from the representative preparation used in Figs. 1—3. For comparison, the average values for the Sephadex G-200 step for consecutive runs were: 70KU, 609 units/mg, 9% recovery.

Purification step	Volume (ml)	Protein (g)	Total activity (units $\times 10^3$)	Specific activity (units/mg)	Purification (-fold) *	Recovery (%)
Crude extract	28 300	651	792	1.2	1	100
Flow-Through **	(26 300)	(297)	(132)	(0.44)	(—)	(17)
Washes	(37 300)	(117)	(75)	(0.64)	(—)	(9)
1st DEAE-cellulose	13 900	67	350	5.3	4.4	44
2nd DEAE-cellulose	7 770	27	244	9.0	7.5	31
CM-cellulose	8 850	0.508	119	234	195	15
Hollow-fiber concentration	28.2	0.426	102	239	199	12
Sephadex G-200	140	0.102	85	833	694	11

* Starting extract is already ten times concentrated over plasma.

** Flow-through and washes are unused fractions; numbers in parentheses show recoveries.

of a poorly bound contaminant (deep yellow-colored fraction) with minimum loss of α-galactosidase. The amount of DEAE-cellulose was less than that needed to bind all of the protein in the crude extract; in this way the more tightly bound proteins displaced the weakly bound contaminants and improved resolution somewhat. The resulting loss of α-galactosidase in the unadsorbed fraction ranged from 4 to 38% with an average loss of 22%. Elution profiles from the first and second DEAE-cellulose columns are presented in Fig. 1.

Plasma α-galactosidase activity binds to well to CM-cellulose below pH 4.5. Due to the difficulty of producing a linear pH gradient, a pH was chosen at which all of the activity was bound and the enzyme was then eluted from the column by increasing the ionic strength. The gradient was deliberately made very shallow, resulting in a dilution of activity which, when coupled with the low pH, perhaps accounted for the rather poor recovery of this step. It was an acceptable procedure, however, because of the increased purification obtained. The bulk of the contaminating protein, including ceruloplasmin if present in the fraction, was eluted at higher ionic strength than the α-galactosidase (Fig. 2).

The hollow-fiber units were indispensable in providing a rapid, reproducible and non-destructive concentrating capability. Backwashing was essential since a large percentage of the protein was adsorbed to the fibers, especially in the beaker unit where shear forces at the fiber surface are minimal.

The α-galactosidase activity consistently eluted from Sephadex G-200 in the trough between two protein peaks (Fig. 3), the relative heights of which depended upon the size of the pooled fraction from the CM-cellulose column. Several precautions were necessary at this stage to minimize pyrogen contamination of the product. Fractions were collected and were assayed rapidly and then filtered under sterile conditions as soon as possible.

Characteristics of the final product

The total 4-MU-Gal activity purified from 130 kg of Cohn fraction IV-1 was

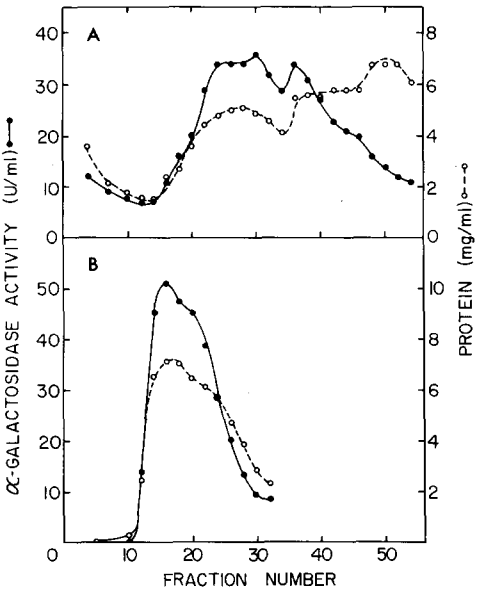

Fig. 1. DEAE-cellulose elution profiles of α-galactosidase. Assays and chromatographic procedures are described under Materials and Methods. The fraction size for both columns was 465 ml. Fractions 14—43 from the first (A) DEAE-cellulose column (15 X 88 cm) were combined and applied to the second (B) DEAE-cellulose column (15 X 18 cm), from which Fractions 13—23 were pooled.

$8 \cdot 10^5$ units at 5630 units/ml. The specific activity was 1450 units/mg protein and the ratio of 4-MU-Gal/$GbOse_3Cer$ activities was 6.2. The sample buffer contained 120 mM NaCl and 25 mM sodium phosphate (pH 6.0). Bacterial endotoxin concentration as determined by the Limulus lysate procedure was 1.0 ng/ml. For immunochemical studies a portion of the final product was concentrated to 76 400 units per ml (50.1 mg protein/ml). Immunoelectrophoresis showed detectable bands in the IgA region with anti-whole serum and with

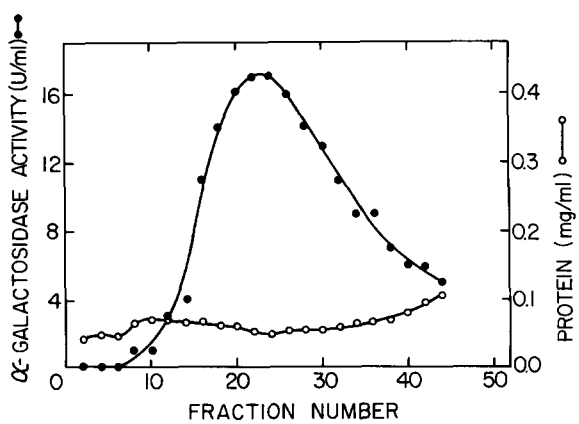

Fig. 2. CM-cellulose elution profile of α-galactosidase. Assays and chromatographic procedures are described under Materials and Methods. The bed height in the 24 cm diameter column was 11.8 cm and the fraction size was 465 ml. Fractions 16—34 were pooled.

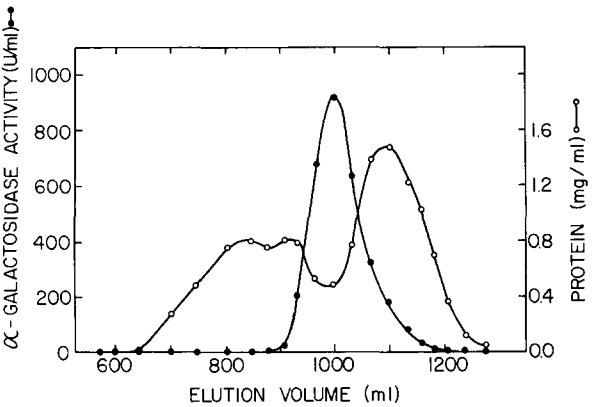

Fig. 3. Sephadex G-200 elution profile of α-galactosidase. Assays and chromatographic procedures are described under Materials and Methods. The fraction size was 15 ml and the flow rate for the 5 X 88 cm column was 21 ml/h. Fractions were pooled from 930 to 1070 ml.

anti-IgA. The unconcentrated final product gave no detectable bands. Using radial immunodiffusion, IgA was barely detectable with the unconcentrated enzyme; with the concentrated enzyme it was present at a concentration of 0.45 mg/ml (0.9%) of the total protein. No precipitin rings were observed for the specific antisera to IgG, IgM, ceruloplasmin, transferrin or albumin, the minimum level of detection being 0.1% of the total protein.

The pyrogen content of the final product was quantitated by the bacterial endotoxin-induced precipitation of protein factors in the lysate from Horseshoe crab amoebocytes [17]. With this test, the endotoxin content of the glass-distilled water used in production was approx. 0.10 ng/ml (compared to the tap water level of 2.13 ng/ml). Serial washing of ten 20-ml test tubes, which had previously been washed by a standard procedure, with 10 ml sterile, pyrogen-free water resulted in a contamination level of 0.13 ng/ml. The pyrogen content of the final product was also estimated in rabbits according

TABLE II

IN VIVO AND IN VITRO PYROGEN DETERMINATIONS

Enzyme preparation	Specific *** activity (units/mg)	Activity *** (units/ml)	Endotoxin † (ng/ml)	Temperature rise in rabbits †† (°C)
1	1040	3310	1.08	0.65
2	1010	4420	0.91	0.56
3	1450	5630	1.00	0.50
3 (Boiled) *	—	—	0.83	—
Albumin-1 **	—	—	0.29	— †††
Albumin-2 **	—	—	0.51	— †††

* 5 min at 100°C supernatant (clinical centrifuge).
** Two sterile lots of commercial human serum albumin.
*** 4-MU-Gal activity, assays described in Materials and Methods.
† Limulus lysate techniques (see Materials and Methods).
†† Average of three rabbits, dose equals 2000 4-MU-Gal units per kg rabbit.
††† Not determined, but must be equal to or less than 0.5°C to pass the test.

TABLE III

STABILITY OF PURIFIED α-GALACTOSIDASE

The stability values for preparation 4 were calculated from the least-squares fit of 9-point stability profiles, using samples from 0 to 139 days. Assay procedures are described in Materials and Methods.

Enzyme preparation	Specific activity (units/mg)	pH 6 buffer	(mM)	Storage temperature (°C)	Activity (units/ml) *					
					4-MU-Gal			$GbOse_3Cer$		
					0 day	10 day	120 day	0 day	10 day	120 day
4	660	Na_2HPO_4/ NaCl	25 / 150	4	953	935	917	—	—	197
4	660	Na_2HPO_4/ NaCl	25 / 150	−20	948	925	903	—	—	202
5	1350	Na_2HPO_4	10	4	650	617	—	120	118	—

* Activity remaining after specified days of storage.

to the official protocol of the U.S. Pharmacopeia test for pyrogens. The two tests gave comparable results (Table II).

The purified final product was stored at 4°C for up to 4 months before use. The stability of various preparations is given in Table III.

The activity of a more thermally stable form of α-galactosidase activity, called α-galactosidase B [3,18], has been shown to be chromatographically and

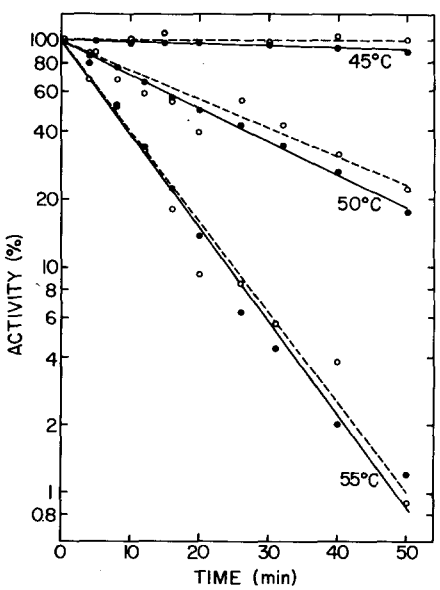

Fig. 4. Heat inactivation of α-galactosidase. The enzyme used was purified through the second Sephadex G-200 step and contained 116 units/ml (4-MU-Gal), 23 units/ml ($GbOse_3Cer$) and 0.10 mg protein/ml in 25 mM sodium phosphate buffer, pH 6.0 and 20 mM NaCl. This enzyme solution was incubated at the stated temperatures and aliquots were chilled at 0°C at the indicated times. All aliquots were assayed for 4-MU-Gal (●———●) and $GbOse_3Cer$ (○———○) activities according to the procedures in Materials and Methods.

immunologically identical with α-*N*-acetylgalactosaminidase [19,20]. Assays of the final product and a similar product from earlier preparations with the *p*-nitrophenyl-α-*N*-acetylgalactosaminide assay of Dean and Sweeley [10] showed that the B form of α-galactosidase was not detectable.

Enzyme which had been purified through the second Sephadex G-200 step was rapidly inactivated at 55°C, with a half-life of about 7 min (Fig. 4).

The purified enzyme was inhibited by *myo*-inositol, a characteristic property of α-galactosidase A [21]. Inhibition by 500 mM *myo*-inositol was 37% at 4.3 mM substrate and 54% at 0.6 mM substrate.

The ratio of activities of the purified plasma α-galactosidase towards 4-MU-Gal and the glycolipid substrate ($GbOse_3Cer$) was nearly identical with that observed with a preparation from human liver [22] and one from placenta [11]. The activities with these two substrates were similarly affected by heat (Fig. 4) and migrated to the same position on polyacrylamide gels. It is therefore concluded that these activities are probably derived from the same protein.

Discussion

This report demonstrates the feasibility of purifying substantial amounts of α-galactosidase A from human plasma. The 9% yield is low, requiring large amounts of Cohn fraction IV-1 to be used as starting material. In comparison, Mayes and Beutler [23] achieved a 36 000-fold purification with an 18% yield from human placenta. The importance of the purification from plasma derives from the observations that plasma α-galactosidase A has a different isoelectric point than that of liver and spleen (Bishop, D.F, and Sweeley, C.C., unpublished data) and has a longer circulatory half-life than the tissue enzyme on infusion into Fabry patients (Desnick, R.J., Dean, K.J., Grabowski and Sweeley, C.C., unpublished data).

It is difficult to determine the precise purity of the α-galactosidase in this preparation. Since the enzyme has not been obtained previously in a homogeneous form from plasma by this isolation procedure, estimates of purity must be based on gel electrophoresis or comparison of the specific activity with that of homogeneous enzyme from another source. We have based our estimates on the latter method, using specific activities for placental enzyme [11,24] and liver enzyme [10]. The specific activities of these tissue preparations varied from 9800 to $1.0 \cdot 10^6$ nmol per mg protein per h (4-MU-Gal activity). By comparison our preparation was in a range from 0.1 to 10% pure, assuming the plasma and tissue specific activities in pure form are the same. Obviously this may not be the case as the plasma and tissue enzymes have different chemical (Bishop, D.F. and Sweeley, C.C., unpublished data) and physiological (Desnick, R.J., Dean, K.J., Grabowski and Sweeley, C.C., unpublished data) properties. The estimate is therefore only a crude approximation.

The production procedure, including Cohn fractionation, resulted in approx. 7000-fold purification of α-galactosidase activity, based on the specific activities of whole plasma and the final product with the 4-MU-Gal substrate. The nature of the remaining contaminants is not yet known. The lack of antigenic response suggests that the bulk of the protein may represent a number of substances whose concentrations in serum are too low to induce antibodies in rab-

bits. A fraction of the product may also be accounted for if the isolation procedure results in some inactivation (not denaturation) of the enzyme.

The availability of a quantitative in vitro pyrogen test was very useful for determining sources of contamination during production. It was important to minimize the levels of pyrogen throughout the process as it was not possible to remove them from the final product. The Sephadex G-200 column separated some high molecular weight pyrogenic material, as observed by others [25], but a substantial proportion remained with the lower molecular weight fractions containing α-galactosidase. The remaining pyrogenic material was found to comigrate with α-galactosidase on a sucrose density gradient (Fig. 5), suggesting that the pyrogen was about the same size, bound to the enzyme, or the enzyme itself. The latter is unlikely since heat precipitation of the enzyme resulted in most of the pyrogen remaining active in the supernatant (Table II).

The plasma α-galactosidase obtained by this procedure is the A form according to the criteria of heat lability, catalytic activity toward globotriglycosylceramide, absence of any α-*N*-acetylgalactosaminidase activity, and inhibition by *myo*-inositol. Most of its chemical and kinetic properties (Bishop, C.F. and Sweeley, C.C., unpublished data) are similar to those of α-galactosidase A from tissues [10,24,26] but its isoelectric point is decidedly lower. Until the reasons for this difference are clearly understood, the plasma enzyme should be differentiated in some way; we have referred to it as the plasma form of α-galactosidase A.

This is the first report of the preparation of the plasma form of α-galactosidase A on a scale that is sufficient to support clinical studies of enzyme replacement therapy in patients with Fabry's disease with this enzyme. The product has excellent long-term stability at 4 or −20°C, making it possible to consider preparation of sufficient amounts of the enzyme for long-term therapeutic trials. Significant improvements in both yield and purity are possible, should the clinical studies indicate that management of the disease is practical by this approach.

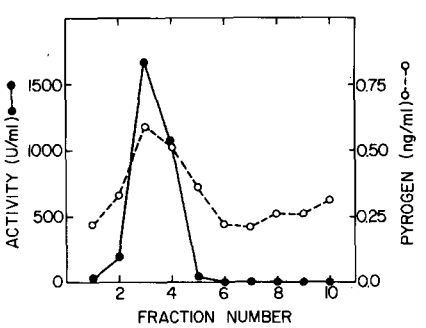

Fig. 5. Sedimentation of pyrogen and α-galactosidase in a sucrose density gradient. The α-galactosidase used was purified to about 1000 units/mg protein (4-MU-Gal) and was approx. 2200 units/ml. The 5—25% sucrose gradient (3.5 ml) was prepared with sterile saline and was layered with 0.5 ml of this enzyme solution. Centrifugation proceded at 40 000 rev./min for 15 h in a Beckman (Palo Alto, Calif.) 50.1 Ti swinging bucket rotor. Approx. 0.3-ml fractions were collected. The assays for α-galactosidase and pyrogen are described in Materials and Methods.

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